



A CHEMICAL-BIOLOGICAL STUDY OF *ESCHERICHIA COLI* AND THREE OF ITS ROUGH VARIANTS¹

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While working on the general subject of filtrability, certain variants of *Escherichia coli* were isolated which are referred to in this paper as *E. coli*-R-27, *E. coli*-R-32, and *E. coli*-R-G. The normal smooth parent strain is designated as *E. coli*-S. Each of the variant organisms formed a colony which was rough in appearance and grew in a characteristic manner when inoculated into broth. The letter "G" signifies only that the colony is small. The rate of multiplication of the parent and variant forms was determined to be the same.

The first section of the paper deals with the ability of these four forms to utilize the salts of organic acids as sources of energy. This is followed by a comparative study of the decomposition of glucose, and of the unique behavior of one of the variants in respect to this carbohydrate. The results of these experiments suggested the work which is expressed in the glucose utilization curves. The effect of the organism when injected into the animal body is also tested, and a sound explanation for the mechanism of action is given.

UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE PARENT CULTURE AND THE VARIANT FORMS

Using a synthetic medium, the ability of the four forms of the colon bacillus to utilize the carbon of certain organic acids as a

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source of energy was studied comparatively. Terminal acidity was measured by the buffer system of Kolthoff (1925). Koser (1923) has conclusively proved that no growth will take place without a source of carbon such as an organic acid, glucose, or lactose. Pesch (1921) employed sodium tartrate and showed that five proven strains of *E. coli* grew well on it, but when sodium citrate was substituted, the same strains showed no growth even after 72 hours. Results are tabulated in table 1.

TABLE 1

Growth, gas production, and final pH of E. coli-S and variants cultured on a synthetic medium containing the sodium salts of organic acids

ACID	E. COLI-S		E. COLI-R-27		E. COLI-R-32		E. COLI-R-G	
	Growth	Final pH	Growth	Final pH	Growth	Final pH	Growth	Final pH
Formic.....	2+	8.0	2+	8.2	2+	8.2	2+	8.2
Acetic.....	4+	7.8	1+	6.6	2+	6.8	1+	6.7
Propionic.....	2+	7.6	1+	7.6	2+	7.6	1+	7.6
Butyric.....	1+	7.6	1+	7.6	1+	7.6	1+	7.6
Lactic.....	v. h.*	8.2	2+	8.0	3+	7.6	2+	7.6
Malic.....	3+	8.2	2+	8.2	1+	8.2	1+	8.2
Oxalic.....	1+	7.5	b. t.*	7.5	1+	7.6	1+	7.6
Valerianic.....	b. t.	7.6	1+	7.6	2+	7.6	1+	7.6
Tartaric.....	1+	7.5	2+	7.6	2+	7.5	2+	7.5
Citric.....	1+	7.5	1+	7.6	2+	7.6	2+	7.6
Benzoic.....	1+	7.5	1+	7.6	1+	7.5	1+	7.5
Salicylic.....	b. t.	7.4	b. t.	7.5	2+	7.5	1+	7.5

Initial pH was in each instance 7.6.

Proliferation was observed in Erlenmeyer flasks.

The Smith fermentation tube was used to estimate gas production.

No gas was formed.

* v. h. = very heavy; b. t. = barest trace.

Table 1 shows marked differences in ability to use the carbon of organic acids as a source of energy in the cases of acetic, lactic, malic, tartaric, citric and salicylic acids. With the first three acids the parent organism showed the greatest development, this being especially true of lactic acid. The last three were more readily utilized by one or more of the variants.

ACTION OF *E. COLI*-S AND THE THREE VARIANTS ON GLUCOSE
AND LACTOSE

The production of acid and gas by the four forms was compared in broth, initial pH 7.6, containing 2 per cent of glucose; a parallel series contained the same amount of lactose. The volumes of gas formed are expressed in centimeters measured from the tops of the closed arms of fermentation tubes, uniform in size. The experiment was done in triplicate, readings being made at the end of 24, 48, and 72 hours. The final pH was 5.2 in all cases where growth occurred. The data are recorded in table 2.

TABLE 2
Gas production in sugar broths
(Heights in centimeters of gas columns in Smith tubes)

BROTH	TUBE	CULTURE											
		S			R-27			R-32			R-G		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Glucose.....	1	5.0	6.5	7.0	3.0	3.7	3.7	0	0	0	0.5	3.8	3.8
	2	5.5	6.0	6.3	—	—	—	0	0	0	2.0	4.6	4.8
	3	4.7	5.8	6.1	0.5	1.5	2.0	0	0	0	0.5	3.5	4.0
Lactose.....	1	1.6	2.5	3.0	*	0.3	1.1	*	*	1.2	0	0	0
	2	4.9	5.3	5.5	*	0.3	1.0	*	*	0.3	0	0	0
	3	4.0	4.5	4.8	*	0.5	1.7	*	0.3	2.0	0	0	0

— = no growth, due probably to faulty inoculation; 0 = heavy growth but no gas; * = first evidence of gas.

These results show that in broth containing glucose *E. coli*-R-32 formed no gas in contrast to the normal S form and the other variants. In broth containing lactose, *E. coli*-R-G formed no gas while the other two variants produced much less than the parent strain. The non-production of gas in glucose broth by *E. coli*-R-32 was studied further.

FURTHER STUDY OF THE ACTION OF *E. COLI*-R-32 ON GLUCOSE

Modification in the gas-producing power of *E. coli* through chemical means has been noted by Penfold (1911), Harden and

Penfold (1912), and Revis (1912). Through the use of sodium chloracetate, Grey (1913) produced a strain unable to form acetaldehyde from glucose. Lommel (1926) has recorded the loss of ability of this bacillus to attack lactose as the result of the action of malachite green. Pakes and Jollyman (1901) proved formic acid to be the immediate precursor of carbon dioxide, more than 80 of their strains being able to form carbon dioxide through the decomposition of this acid.

Inoculation of the four organisms was made into fermentation tubes containing (1) plain broth, (2) 2-per-cent glucose broth, (3) peptone water containing sodium formate and (4) plain broth

TABLE 3

Growth and gas production by E. coli-S and variants in glucose and sodium formate broths and peptone water

	CULTURE							
	S		R-27		R-32		R-G	
	Growth	Gas production	Growth	Gas production	Growth	Gas production	Growth	Gas production
Plain broth.....	+	0	+	0	+	0	+	0
Glucose broth.....	+	+	+	+	+	0	+	+
Na formate peptone water.....	+	+	+	sl.*	+	0	+	sl.
Na formate broth.....	+	+	+	+	+	0	+	sl.

* sl. = slight amount.

containing 1-per-cent Na formate. The following is the composition of the peptone water: (a) 2 per cent peptone, (b) 0.5 per cent sodium formate, (c) 1 per cent sodium formate. After sterilization and incubation for sterility, 8 tubes were inoculated in duplicate with each of the four strains. The readings were taken at the end of 72 hours, the results being recorded in table 3.

A careful analysis of the preceding data will show that those organisms which were able to form gas from glucose broth also formed it from the media containing sodium formate, although not necessarily to the same extent. The one strain which was thoroughly consistent in its action was *E. coli*-R-32.

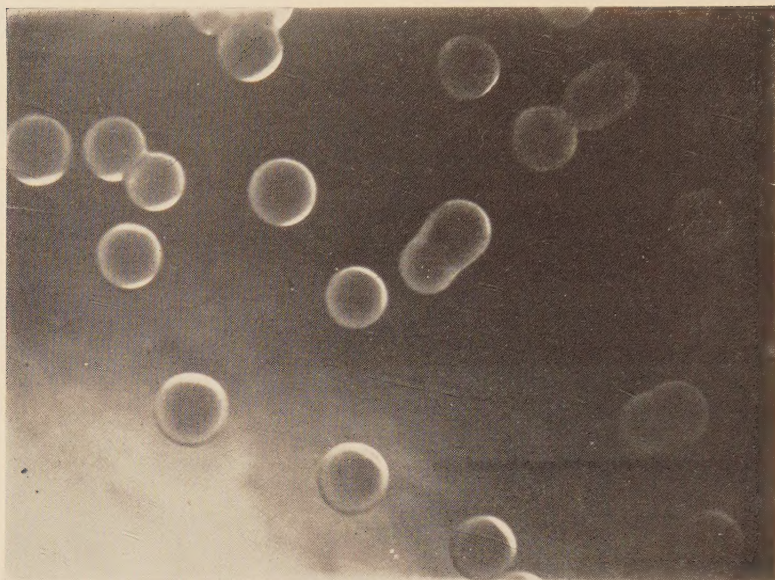
With this information, the next step was to develop a technique for the analysis of formic acid, and the method of titration with KMnO_4 (Ost and Klein, 1908) was selected. Preliminary experiments showed that acetic acid caused no interference in the range studied.

Technique of the experiment. Six flasks were prepared in the following manner: 10 grams of Witte's peptone were boiled with tap water, and after cooling, 20 grams of glucose and 10 grams of pure calcium carbonate were added. The volume was then made up to 1 liter, after which the resulting mixture was sterilized at 110°C . for 30 minutes. This is essentially the medium used by Harden (1901) under somewhat similar conditions. After incubation for sterility, these flasks were inoculated in triplicate with *E. coli*-S and *E. coli*-R-32. At the end of 4 days' incubation at 38°C . the flasks were analyzed in the following manner:

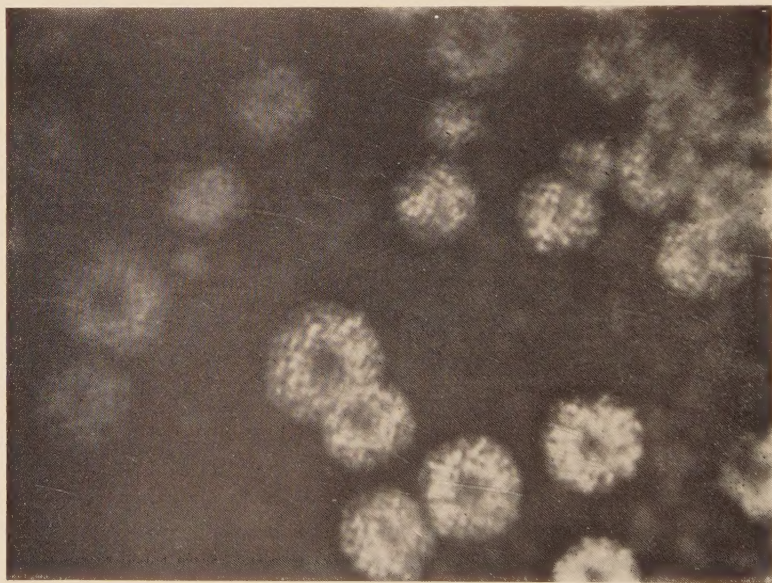
The contents of a flask was first acidified with concentrated H_2SO_4 , and then distilled to carry over volatile acids such as formic and acetic. The last portion was steam-distilled until tests showed that no further acids were coming over. The distillate was then made alkaline with sodium carbonate solution, evaporated to a convenient volume, and finally titrated hot with $\text{N}/10$ KMnO_4 . The absence of higher acids such as butyric and propionic was indicated by qualitative tests. The titration data follow:

	Volume of $\text{N}/10$ KMnO_4 used cc.
1. <i>E. coli</i> -S.....	56.10
2. <i>E. coli</i> -S.....	57.55
3. <i>E. coli</i> -S.....	56.75
1. <i>E. coli</i> -R-32.....	114.35
2. <i>E. coli</i> -R-32.....	114.90
3. <i>E. coli</i> -R-32.....	113.85

This experiment shows that, even in the case of the S form of the organism, there is undecomposed formic acid when growth of the germs has ceased, but that in the case of the rough variant there is much more. It seems incontrovertible that in the case of *E. coli*-R-32 the reason for its failure to produce gas from glucose

FIG. 1. *E. COLI-S*

All photographs taken at magnification $10\times$ from plain agar plates, 36 hours incubation.

FIG. 2. *E. COLI-R-27*

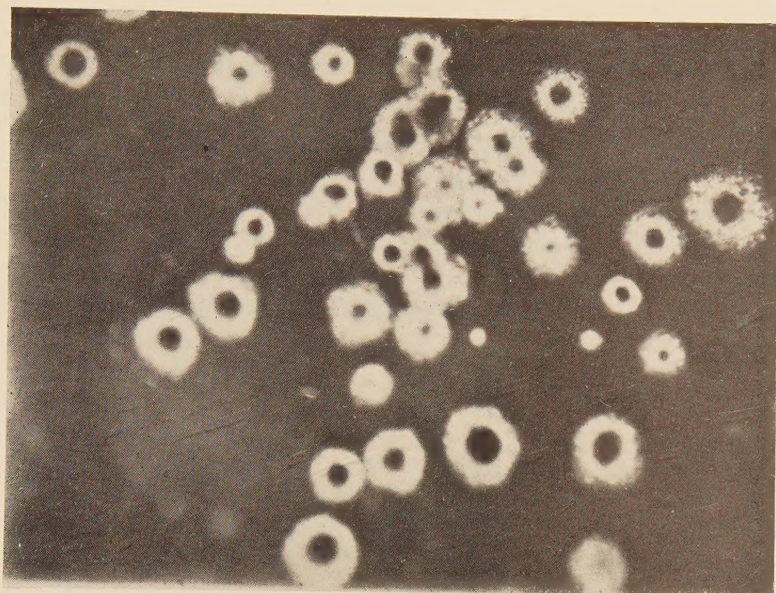


FIG. 3. *E. COLI*-R-32

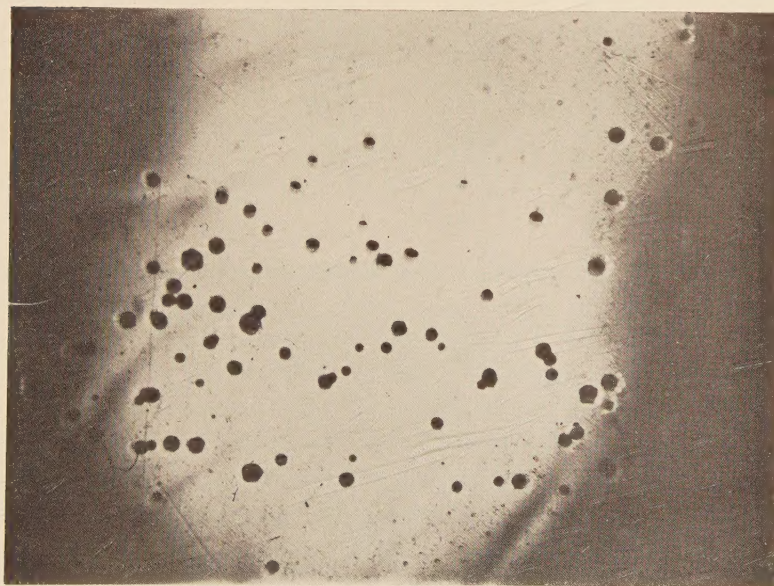


FIG. 4. *E. COLI*-R-G

is its inability to utilize formic acid. Penfold (1911) worked extensively on the general relation of the power of micro-organisms to form gases and their ability to decompose formates. He isolated a modified strain of *E. coli* by growth in the presence of sodium mono-chloracetate, and demonstrated that this variant formed acid but no gas from glucose, fructose, and lactose, although it still yielded gas from the alcohols used and from formates. Harden and Penfold (1912) later worked with this modified strain, proving that the main difference between the actions of the strains lay in the end products of fermentation,

TABLE 4

The utilization of glucose by E. coli-S when added to nutrient broth in various concentrations (average values)

NUMBER OF TESTS	VOLUME OF BENEDICT'S SOLUTION USED	ORIGINAL CONCENTRATION OF GLUCOSE	VOLUME OF FILTRATE USED FOR GLUCOSE DETERMINATION	FINAL CONCENTRATION OF GLUCOSE	GLUCOSE UTILIZED
	cc.	mgm. per cc.	cc.	mgm. per cc.	per cent
5	25.00	55.60	0.90	55.60	00.00
5	25.00	45.07	1.17	42.78	5.04
5	25.00	31.25	1.82	27.48	12.06
5	25.00	23.04	2.67	18.74	18.71
5	25.00	13.77	4.85	10.31	25.13
5	25.00	10.10	7.66	6.53	35.37
2	55.00*	6.50	2.00	3.23	50.35
2	55.00*	5.12	3.00	1.13	78.02

* Bang's solution used.

those from the modified strain containing less hydrogen and carbon dioxide and more formic acid than those from the normal strain. The atypical strain also made more lactic² acid than the normal strain and less alcohol and acetic acid.

GLUCOSE UTILIZATION BY *E. COLI-S* AND *E. COLI-R-27*

The utilization of glucose by *E. coli-S* and *E. coli-R-27* was studied in a broth solution containing different amounts of the

²Neuberg and Gorr (1925) state that "lasst man das *Bacterium coli* auf Methylglyoxal einwirken, so Erfolgt, wie gesagt, die Umwandlung in Milchsäure quantitativ." This should be tested with respect to the variants of *E. coli*.

carbohydrates (tables 4 and 5). The concentrations of the solutions are expressed in milligrams of glucose per cubic centimeter, and these values are plotted (in fig. 5) against the per cent of glucose utilized by the organism. The concentrations of sugar ranged from those so low that fermentation was practically complete to values so high that no measurable amount was decomposed. Benedict's method of analysis was used when practicable, but in the extremely low dilutions this was replaced by the micro-method of Bang (1913). A uniform correction for evaporation, previously determined by analysis of a large number of samples, was applied.

TABLE 5

The utilization of glucose by E. coli-R-27 when added to nutrient broth in various concentrations (average values)

NUMBER OF TESTS	VOLUME OF BENEDICT'S SOLUTION USED	ORIGINAL CONCENTRATION OF GLUCOSE	VOLUME OF FILTRATE USED FOR GLUCOSE DETERMINATION	FINAL CONCENTRATION OF GLUCOSE	GLUCOSE UTILIZED
	cc.	mgm. per cc.	cc.	mgm. per cc.	per cent
5	25.00	62.5	0.85	58.8	3.8
5	25.00	41.66	1.26	39.56	4.27
3	25.00	27.03	2.02	24.87	8.00
3	25.00	17.86	3.36	14.88	16.66
3	25.00	12.50	5.48	9.12	27.00
2	55.00*	8.92	1.50	3.52	60.36
2	55.00*	5.83	2.00	0.86	85.35

* Bang's solution used.

BIOLOGY AND BIOLOGICAL APPLICATIONS

A. Effect on the blood sugar of the injection of E. coli-S and the three rough variants

In this experiment, the term blood sugar means reducing sugar or glucose. Zeckwer and Goodell (1925) injected killed cultures of *E. coli* suspended in physiological salt solutions into rabbits *via* the ear vein, and found that the content of blood sugar began to rise almost immediately, reaching a maximum in less than two hours and returning to normal in about four hours. Later (1925) the same authors proved that during bacterial

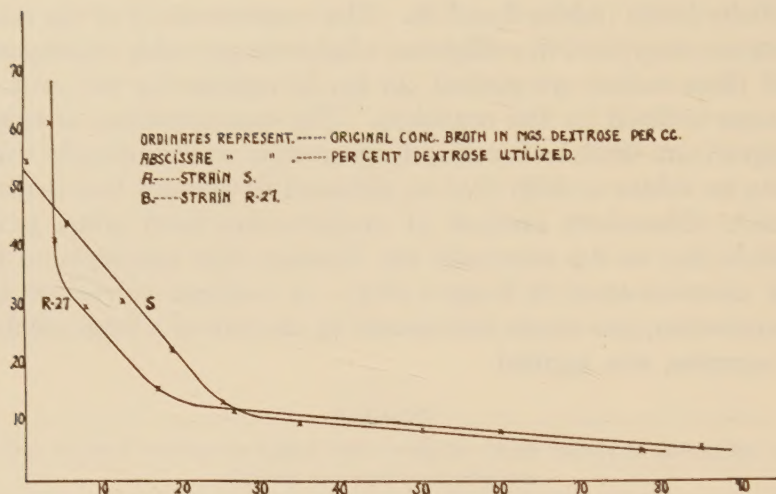


FIG. 5. GLUCOSE UTILIZATION CURVES

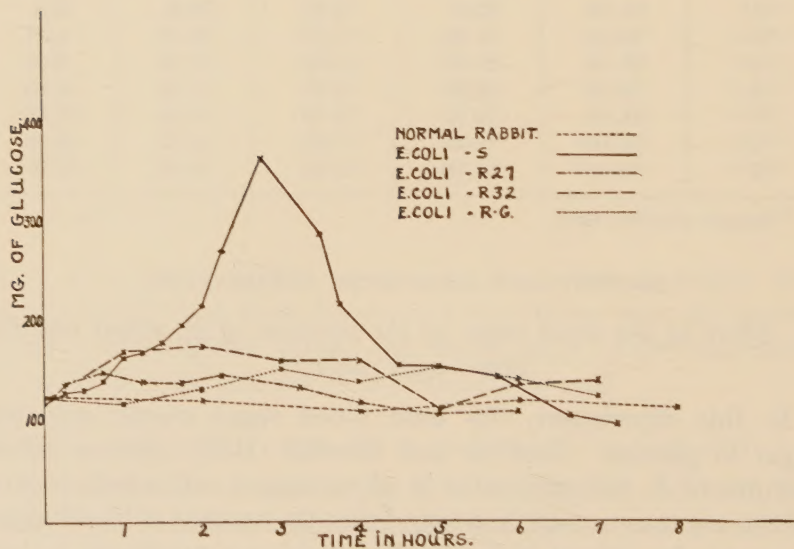


FIG. 6. MILLIGRAMS OF GLUCOSE PER 100 CC. BLOOD

anaphylaxis there was a gradual rise in the blood sugar which attained an extremely high level at the time of death. Delafield (1932) obtained practically identical results.

The procedure was to inoculate into the ear vein of a rabbit a certain amount of the young broth culture of the organism. The basis of the amount was enough of *E. coli*-S to cause the death of the animal in 24 hours. An equal amount of the other organ-

TABLE 6

Effect on the blood sugar of rabbits of the injection of broth cultures of E. coli-S and variants

INTERVAL AFTER INJECTION	NORMAL CONTROLS		COLI-S	R-27	R-32	R-G
	1*	2*				
<i>minutes</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
00	138	117	120	117	127	127
15			124	134		
45			146	150		
60			170		177	122
75			172	141		
105			199	141		
120	148	101	221		180	138
135			276	150		
165			374			
180					166	157
240	127	101		122	166	143
270			159			
300			159	108	119	157
360	118	134		113	141	145
420					146	127
435			102			
480	101	134				

* Average is plotted as one curve.

isms was used, although it had been demonstrated many times that the variant forms are entirely harmless for rabbits. For the measurement of the blood sugar, the method of Hagedorn and Jensen (1922-23) was used. The data are presented in both tabular and graphic form.

These results show a marked difference in the reaction of the animal body to the S and R forms. Within three hours of the

injection of the S form, the blood sugar content had risen from 120 to 372 mgm. per 100 cc. of blood. Concurrently with this change, the rabbit became increasingly restless and died the same evening.

The reactions to the variants show considerable similarity and suggest a close relationship between the R forms. The slight increases in blood sugar are certainly due to the injection and not to normal variation, for a comparison with the two normal rabbits shows that this value is not changing greatly. None of the rabbits which received the injection of any of the variant forms showed any ill effects at any time.

It is therefore evident that the increase in the blood sugar must be due to a toxin formed by the normal S form and not at all, or at most in very small amounts, by the rough variants. Death of the animal in this, and more especially in other instances in which larger amounts of the broth culture have been used, has occurred too quickly to be ascribed to multiplication of the bacteria.

B. Pathogenicity for animals of E. coli-S and the three rough variants

The fact has been established that the parent organism when injected intravenously into rabbits produces death in a very short time, while similar injections of the other three forms leave the animals apparently unaffected. Through the injection of filtrates as well as of washed masses of cells which had been frozen and thawed repeatedly, it was conclusively demonstrated during this work that the lethal agent is an exo-toxin. In the dog, Denys and Sluyts (1894) proved the toxin of *E. coli* to have an intense action on the mucosa of the digestive tract. Roger (1894), using graphical methods, showed that the toxin of this organism made the heart less irritable. Vida (1905) believed that *E. coli* possessed only an endo-toxin while Vincent (1925) insisted that it possessed both an endo- and an exo-toxin. Steinberg and Ecker (1926) obtained from young cultures a toxic substance which proved to be the lethal factor when injected into rabbits. Smith (1927) stated that the offensive weapon of the organism appears to be a soluble, diffusible toxin.

In order to determine the manner in which the toxin of the normal S form caused death in rabbits, many of these animals were autopsied and the organs examined microscopically. The picture was invariably the same—extensive pigmentation in the liver but with the real significant pathology to be found in the lung. Here, the normal lung structure was almost obliterated, and the few alveolar spaces which remained were tense and distorted. These spaces were greatly dilated and engorged with red and white blood cells, giving the appearance of extensive hemorrhage. Many spaces had been obliterated by the presence of the blood cells in them as well as by pressure from the outside. Death was due to the fact that the respiratory membrane of the lung had been made suddenly permeable to the blood cells through the action of the bacterial toxin. This explains the death of the animal with symptoms of marked dyspnoea. This matter warrants much further investigation.

SUMMARY

Three rough variants of *Escherichia coli* have been developed which possess marked differences in comparison with each other and with the parent strain. As a source of energy, acetic, lactic and malic acids are more readily utilized by the parent cell, while tartaric, citric and salicylic acids are more readily utilized by one or more of the variants.

In broth containing two per cent glucose, one of the variants, *E. coli*-R-32, is unique in that there is an absence of gas formation with an end reaction which is acid. This is proved to be due to the inability of this cell to decompose formates.

With respect to the decomposition of glucose, it is demonstrated that *E. coli*-R-27, the most typical of the rough variants, has a less vigorous enzyme, or set of enzymes, than the parent cell.

The difference in the action of the normal strain and the variants on the animal body is marked. The injection of a lethal dose of *E. coli*-S into the ear vein of a rabbit causes a sudden rise in the blood sugar, and at the same time brings about the death of the animal in a few hours. The variants under identical conditions are almost without effect.

It is believed that the fatal agent in the action of the normal form is a soluble exo-toxin which produces death by making the walls of the lung capillaries suddenly permeable to the cellular and non-cellular elements of the blood.

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